

Ku70, a Component of DNA-Dependent Protein Kinase, Is a Mammalian Receptor for *Rickettsia conorii*

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SUMMARY

Rickettsia conorii, a strictly intracellular and category C priority bacterial pathogen (NIAID), invades different mammalian cells. Although some signaling events involved in bacterial entry have been documented, the bacterial and host proteins mediating entry were not known. We report the identification of the Ku70 subunit of DNA-dependent protein kinase (DNA-PK) as a receptor involved in *R. conorii* internalization. Ku70 is recruited to *R. conorii* entry sites, and inhibition of Ku70 expression impairs *R. conorii* internalization. Bacterial invasion is dependent on the presence of cholesterol-enriched microdomains containing Ku70. *R. conorii* infection stimulates the ubiquitination of Ku70. In addition, the ubiquitin ligase c-Cbl is recruited to *R. conorii* entry foci, and downregulation of endogenous c-Cbl blocks bacterial invasion and Ku70 ubiquitination. An affinity chromatography approach identified the rickettsial protein rOmpB as a ligand for Ku70. This is the first report of a receptor-ligand interaction involved in the internalization of any rickettsial species.

INTRODUCTION

Rickettsiae are obligate intracellular pathogens that are transmitted to human hosts through arthropod vectors (Hackstadt, 1996). Rickettsiae are divided into two groups,

the typhus group (TG) and the spotted fever group (SFG), based on differences in their antigenicity to lipopolysaccharide and outer-membrane proteins and on the differences in the diseases that they cause (Vishwanath, 1991). Members of both groups are responsible for severe human diseases (Hackstadt, 1996) and have been classified as select agents for potential use as tools for bioterrorism by the National Institute of Allergy and Infectious Diseases (NIAID) (http://www2.niaid.nih.gov/biodefense/bandc_priority.htm). The genomes of several rickettsial species, including *Rickettsia prowazekii*, the causative agent of epidemic typhus (Andersson et al., 1998), and *Rickettsia conorii*, the causative agent of Mediterranean spotted fever (Ogata et al., 2001), have been sequenced; however, genetic tools are lacking, thereby limiting the use of sequence data.

Some SFG rickettsiae, including *R. conorii*, are transmitted by tick bite inoculation into the skin of the human host (Hackstadt, 1996), and subsequent growth in endothelial cells can result in a localized dermal and epidermal necrosis called eschar or tache noire (Walker et al., 1988). Further injury to the vascular endothelium and infiltration of perivascular mononuclear cells leads to an increase in fluid leakage into the interstitial space and ultimately results in a characteristic dermal rash (Hand et al., 1970; Walker et al., 1988). *R. conorii* invades a variety of different cell types and spreads either via lymphatic vessels to lymph nodes or via the bloodstream to various tissues (Walker and Gear, 1985). Within the cytoplasm of infected cells, *R. conorii* propels itself intra- and intercellularly using the bacterial RickA protein to polymerize host actin filaments (Gouin et al., 2004; Jeng et al., 2004). Infections are controlled by broad-spectrum antibiotic therapy; however, if left untreated, Mediterranean spotted fever can result in severe morbidity and mortality (Yagupsky and Wolach, 1993).

We recently demonstrated that invasion of mammalian cells in vitro by *R. conorii* is mechanistically similar to that of other established invasive pathogens and depends on actin polymerization controlled by the Arp2/3 complex (Martinez

and Cossart, 2004; Cossart and Sansonetti, 2004). As with other bacterial diseases, adherence to target cells is a crucial step in establishing a successful rickettsial infection. Although putative bacterial adhesins, namely rOmpA (Li and Walker, 1998) and rOmpB (Uchiyama, 2003), in other SFG rickettsiae have been proposed to mediate adherence to and subsequent internalization in host cells, nothing was known about the cell receptors utilized by SFG rickettsiae to enter cells.

RESULTS

R. conorii Interacts with a Subset of Host Proteins

We utilized a bacterial whole-cell pull-down assay with soluble Vero and HeLa cell proteins to identify host proteins that interact with *R. conorii*. Mass-spectrometry analysis of trypsin-digested peptides from four of these isolated proteins revealed that β -actin, Ku70, Ku86, and poly(ADP-ribose) polymerase (PARP, p113) interacted with *R. conorii* (Figure 1A, arrows; see also Figure S1A and S1B in the Supplemental Data available with this article online). Ku70 and Ku86 along with a 460 kDa catalytic subunit comprise the DNA-dependent protein kinase (DNA-PK), a serine/threonine kinase with homology to the phosphoinositide (PI) 3-kinase superfamily (Hartley et al., 1995). PARP is a nuclear protein that is implicated in DNA repair in response to DNA damage and interacts with Ku70/86 heterodimers in the absence of DNA (Galande and Kohwi-Shigematsu, 1999). Although Ku70 and Ku86 are primarily nuclear, these proteins are also found in the cytoplasm and at the plasma membrane (reviewed in Koike, 2002). Interestingly, Ku70 has been predicted to span the plasma membrane (Chan et al., 1989) and, along with Ku86, can mediate hetero- and homotypic cellular adhesion (Koike, 2002) as well as adherence to fibronectin (Monferran et al., 2004a). We therefore investigated the possibility that plasma-membrane-localized Ku70 and/or Ku86 could serve as a host-cell receptor for *R. conorii*. Vero and HeLa cell-surface proteins were incubated with a membrane-impermeable biotin reagent to differentially label surface proteins, and soluble protein lysates from these cells were utilized in the bacterial pull-down assay. We determined that an approximately 70/75 kDa biotinylated host protein interacted with *R. conorii* but not with *E. coli* or *L. monocytogenes* (Figure 1B). Western immunoblot analysis of elutions from the bacterial pull-down assay with an anti-Ku70 monoclonal antibody (N3H10) revealed that *R. conorii*, but not other invasive pathogens, interacted with membrane-associated Ku70 (Figure 1C).

Ku70 Is Involved in *R. conorii* Invasion of Mammalian Cells

We examined whether the interaction of *R. conorii* with Ku70 was critical for the infection of nonphagocytic mammalian cells. Fluorescence microscopy analysis of *R. conorii*-infected cells first demonstrated that Ku70 is recruited to bacterial entry foci within 15 min after infection of Vero cells (Figure 2A) and HeLa cells (data not shown). To first test whether or not Ku70 serves as a *R. conorii* receptor, we utilized a monoclonal antibody specific to a Ku70 N-terminal epitope (N3H10) previously used for functional blocking

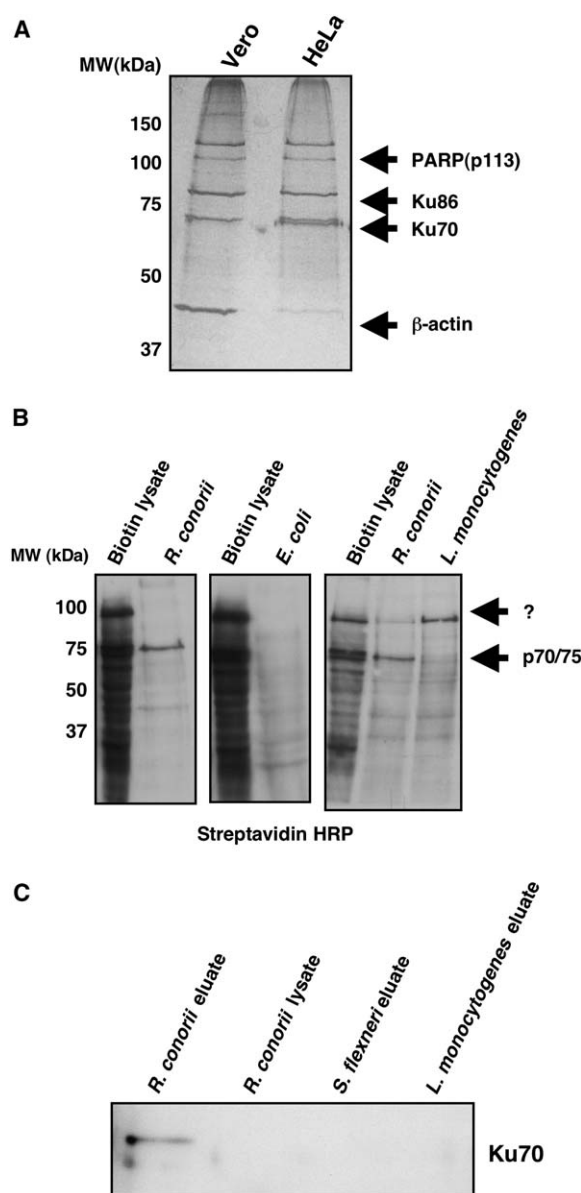


Figure 1. *R. conorii* Interacts with a Subset of Specific Host-Cell Proteins

(A) Eluates from an in vitro bacterial affinity pull-down assay using Vero and HeLa cells were separated on SDS-PAGE and silver stained. Mass-spectrometry analysis of protein bands identified specific host-cell proteins, including Ku70, Ku86, PARP, and β -actin (arrows), that interact with *R. conorii*.

(B and C) Biotin-labeled surface proteins from Vero cells were incubated with *R. conorii* to determine host-cell surface proteins that could potentially serve as receptors. *R. conorii* but not *E. coli*, *L. monocytogenes* (EGD), or *S. flexneri* (M90T) was found to interact with a 70–75 kDa plasma-membrane protein as revealed by immunoblotting with streptavidin-HRP (arrow in [B]) that was subsequently identified by immunoblotting as Ku70 with monoclonal antisera directed at Ku70 (N3H10) (C). Arrow marked “?” in (B) refers to a biotin-labeled surface protein that strongly interacts with *L. monocytogenes* but was not further analyzed. Biotin lysate in (B) refers to the biotin-labeled cell lysate prior to incubation with whole-cell bacteria. *R. conorii* lysate in (C) demonstrates that the interaction with Ku70 is specific and is not due to copurification of Ku70 during isolation of *R. conorii* from mammalian cells.

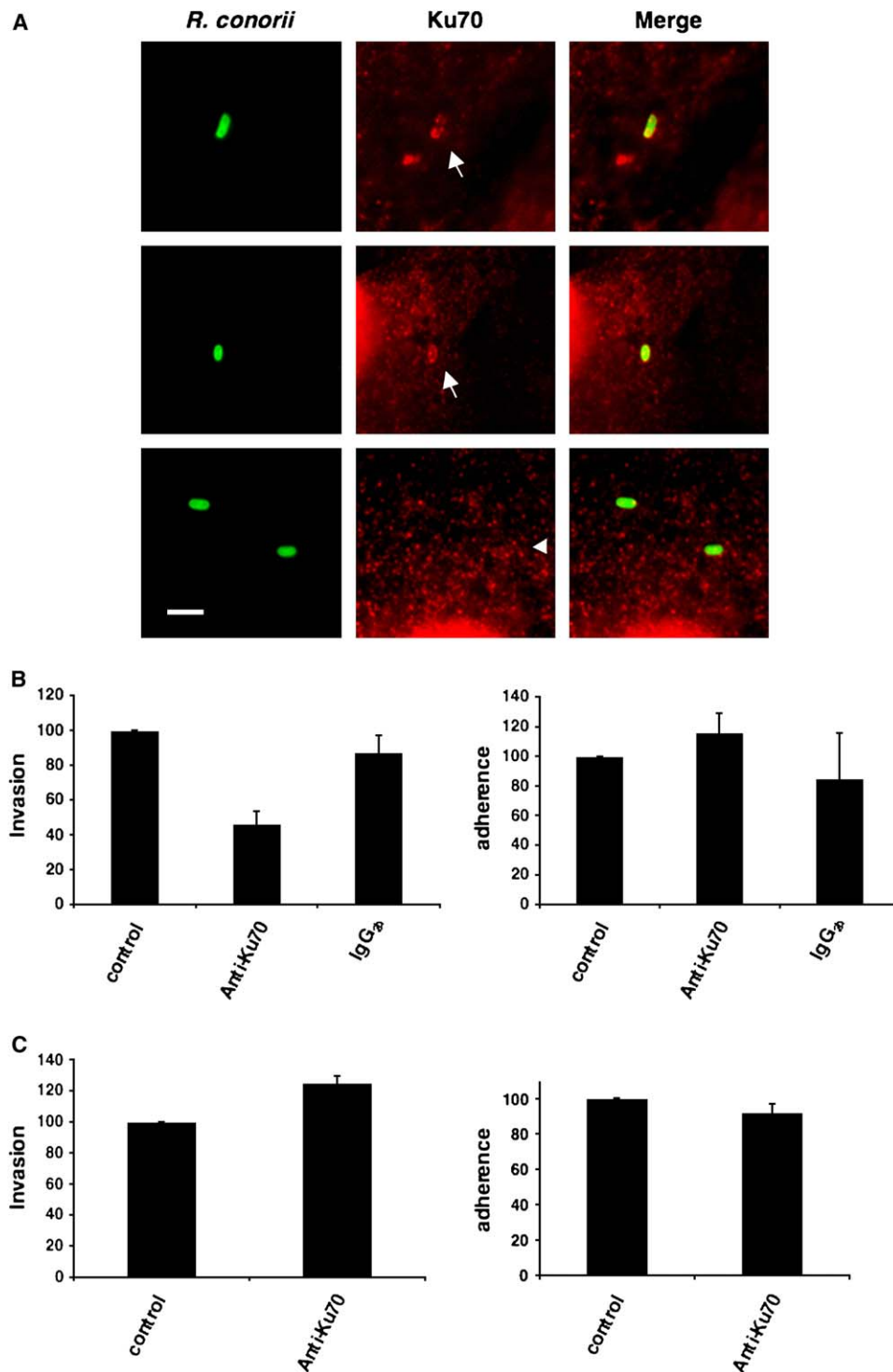


Figure 2. Involvement of Ku70 at the Plasma Membrane as a Receptor for *R. conorii* Invasion

(A) Vero cells were incubated with *R. conorii* for 15 minutes and then processed for immunofluorescence. Extracellular *R. conorii* colocalizes with plasma-membrane-associated Ku70 at sites of bacterial entry in nonpermeabilized cells (arrows, upper and middle panels). In the lower panel, extracellular bacteria are not labeled, demonstrating that labeling is specific and not due to copurification of Ku70 during the *R. conorii* isolation procedure (arrowhead). Bar represents 1 μ m.

(B and C) Antisera directed against an N-terminal epitope of Ku70, but not an isotype-matched monoclonal antibody (IgG_{2b}), is able to efficiently block *R. conorii* invasion of nonphagocytic Vero cells (B). An antibody directed against a predicted cytoplasmic C-terminal epitope of Ku70 that does not recognize Ku70 at the plasma membrane does not affect entry as expected (C). Bacterial-cell association (adherence) is unaffected by either antibody treatment ([B] and [C], right panels). Invasion indices are presented as relative to the control untreated cells (set to 100%).

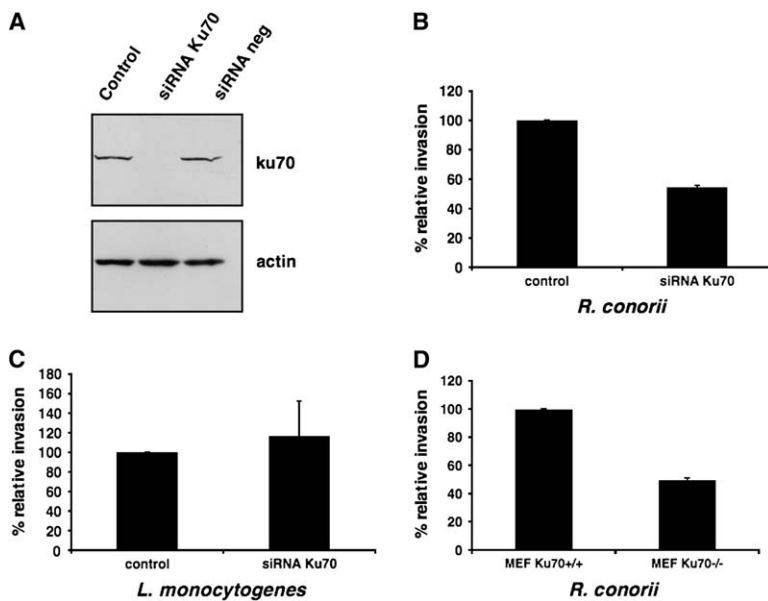


Figure 3. Inhibition of Endogenous Ku70 Expression Blocks *R. conorii* Internalization of Mammalian Cells

(A) siRNA against Ku70 is able to efficiently block Ku70 expression in transfected HeLa cells as determined by immunoblot analysis of cell lysates. Actin was used as a protein loading control. (B and C) *R. conorii* internalization (B) but not *L. monocytogenes* internalization (C) is inhibited in Ku70 siRNA-transfected HeLa cells. (D) *R. conorii* entry is also inhibited in nontransformed mouse embryonic fibroblasts (MEFs) derived from *Ku70*^{-/-} knockout mice as compared to entry into MEFs derived from wild-type littermates. Invasion indices are presented relative to the untransfected control (which was set to 100%).

studies of Ku70 at the plasma membrane (Monferran et al., 2004a). While having no effect on bacterial binding, N3H10 effectively blocked *R. conorii* entry into Vero cells (approximately 55% inhibition relative to control; Figure 2B). An antibody directed against a C-terminal domain of Ku70 (C-19) had no effect on binding or bacterial entry (Figure 2C). Immunofluorescence studies of HeLa cells using these two antibodies confirmed that N3H10 but not C-19 is able to recognize a Ku70 epitope exposed to the extracellular milieu (Figure S2). To confirm that Ku70 is involved in the *R. conorii* entry process, we utilized small interfering RNA (siRNA) against Ku70 to reduce the expression of endogenous total Ku70 protein in HeLa cells (Figure 3A). Suppression of Ku70 expression in HeLa cells effectively blocked *R. conorii* entry to similar levels observed in the antibody inhibition experiments (50% inhibition relative to control; Figure 3B). The entry of *L. monocytogenes* into HeLa cells is dependent on the interaction of the bacterial ligand InlB and its host-cell receptor, the hepatocyte growth-factor receptor, c-Met/HGFR (Cossart et al., 2003). Suppression of Ku70 expression has no effect on *L. monocytogenes* invasion of HeLa cells (Figure 3C).

Expression of Ku70 at the plasma membrane is most often detected in transformed and tumor cell lines of various origin but has also been detected in nontransformed cells (Muller et al., 2005). To address whether Ku70 may have an important role in an in vivo *R. conorii* infection, we performed internalization assays using nontransformed mouse embryonic fibroblasts (MEFs) derived from *Ku70*^{-/-} knockout mice and wild-type littermates. A decrease in *R. conorii* invasion similar to that of siRNA-treated HeLa cells was observed in assays using *Ku70*^{-/-} MEFs (Figure 3D), suggesting that the interaction of *R. conorii* with Ku70 may be important in vivo. Together, these results demonstrate that Ku70 can be utilized by *R. conorii* at the plasma membrane as a receptor mediating bacterial entry.

***R. conorii* Entry Is Cholesterol Dependent, and Ku70 Is Associated with Lipid Microdomains**

The internalization of various viruses, bacteria, and parasites into phagocytic and nonphagocytic cells often involves host membrane microdomains called lipid rafts (Manes et al., 2003; Shin and Abraham, 2001). These domains are abundant in cholesterol, glycosphingolipids, and certain proteins and are implicated in signaling events. In addition, previous reports had identified cholesterol at sites of rickettsial attachment on sheep erythrocyte and other cell membranes (Ramm and Winkler, 1976; Walker et al., 1983). We therefore investigated whether plasma-membrane organization into lipid microdomains is important for rickettsial entry. Because cholesterol is essential for lipid microdomain integrity, drugs that deplete membrane cholesterol and disrupt microdomain organization have been instrumental to study the function of lipid rafts (Kilsdonk et al., 1995). Depletion of membrane cholesterol by methyl- β -cyclodextrin (M β CD) strongly inhibited *R. conorii* entry into Vero cells (Figure 4A) but had no significant effect on bacterial adherence (Figure 4B). Addition of water-soluble cholesterol restored bacterial entry, demonstrating that the inhibition of bacterial entry was due specifically to cholesterol depletion (Figure 4A).

Interestingly, Ku70 has been reported to be localized within discrete microdomains, and it had been speculated that this localization may be important for cellular signaling events (Lucero et al., 2003). A biochemical approach to study lipid microdomains is the isolation of detergent-resistant membranes (DRMs) that are enriched in lipid-raft components. DRMs are resistant to solubilization in cold nonionic detergents and can be isolated in the "low-density" fractions of sucrose gradients (London and Brown, 2000; Mora et al., 1999). We localized a significant portion of Ku70 in the low-density DRM fractions with caveolin-2, a protein known to associate with lipid rafts (Mora et al., 1999) (Figure 4C), confirming previously reported findings (Lucero et al., 2003).

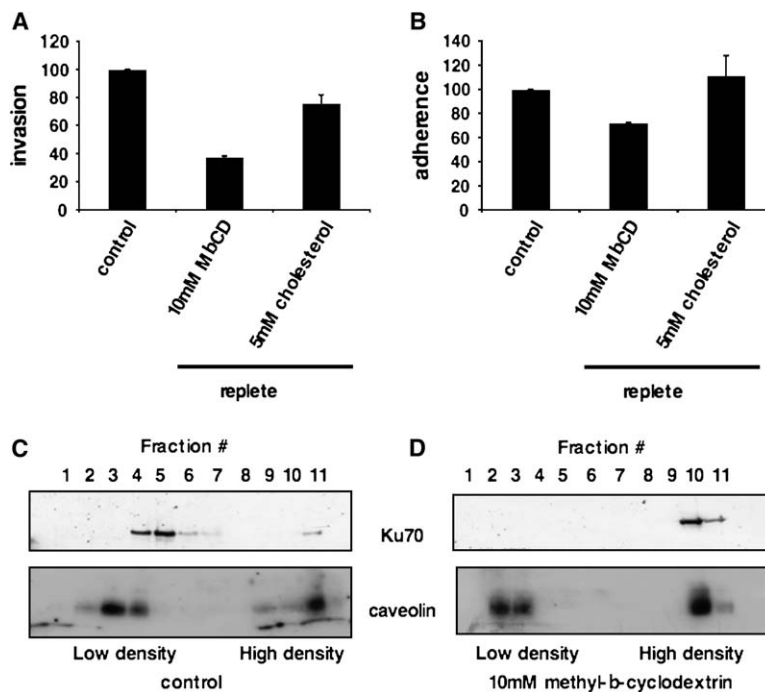


Figure 4. Detergent-Resistant Microdomains Are Important in *R. conorii* Entry of Mammalian Cells

(A) Depletion of host membrane cholesterol with MβCD inhibits *R. conorii* entry into Vero cells, while repletion of these cells with soluble cholesterol restores bacterial invasion.

(B) Depletion of cholesterol does not inhibit the association of *R. conorii* with these cells.

(C and D) Vero cells were processed for isolation of detergent-resistant microdomains (DRMs, or “lipid rafts”) as described in [Experimental Procedures](#). Western blot analysis of isolated proteins localized to low-density and high-density fractions demonstrates the comigration of Ku70 with the raft marker caveolin-2 (C). Depletion of host-cell cholesterol with MβCD disrupts the presence of Ku70 in DRMs as determined by Western immunoblot analysis with Ku70 and caveolin-2 antisera (D).

Treatment of cells with MβCD prior to cold-detergent extraction resulted in the migration of both Ku70 and caveolin-2 in the “high-density” fractions (Figure 4D). Together, these results are in accordance with the hypothesis that Ku70 association with lipid microdomains plays an important role in cholesterol-dependent bacterial entry.

c-Cbl Is Involved in the Ubiquitination of Ku70 and in *R. conorii* Entry

Entry of *L. monocytogenes* into nonphagocytic mammalian cells is in part dependent on the Cbl-mediated monoubiquitination and subsequent endocytosis of c-Met/HGFR, the receptor for a member of the internalin family, InlB (Veiga and Cossart, 2005). Due to some similarities between the entry of *L. monocytogenes* and *R. conorii* into nonphagocytic mammalian cells (Martinez and Cossart, 2004), we first investigated whether ubiquitination of Ku70 occurs during *R. conorii* invasion. HeLa cells were infected with *R. conorii*, and modifications on Ku70 were revealed by immunoprecipitation and Western immunoblotting. Infection of HeLa cells with *R. conorii* induced a rapid ubiquitination of Ku70 in addition to the association of at least one other ubiquitin-modified protein with Ku70 (Figure 5A, arrows). Although modified via ubiquitin, the amount of Ku70 did not change during the course of the infection (Figure 5B).

The ubiquitin ligase c-Cbl is involved in the monoubiquitination of various membrane proteins, including c-Met/HGFR (Petrelli et al., 2002; Soubeyran et al., 2002). In addition, c-Cbl-mediated monoubiquitination and endocytosis of c-Met is critical for *Listeria* entry (Veiga and Cossart, 2005). To initially investigate whether c-Cbl plays a role in *R. conorii* invasion of mammalian cells, HeLa cells were transfected with a plasmid expressing HA-tagged c-Cbl, infected with *R. conorii*, and then processed for immunofluorescence.

R. conorii recruited Cbl to sites of bacterial attachment within the first 3–5 min of infection (Figure 6A). To determine whether Cbl plays a role in *R. conorii* entry, we utilized siRNAs to inhibit the expression of endogenous Cbl in HeLa cells and tested the ability of *R. conorii* to invade these cells. siRNA treatment of HeLa cells efficiently blocked the expression of endogenous Cbl but had no effect on other proteins such as actin (Figure 6B). Inhibition of Cbl expression blocked *R. conorii* entry into HeLa cells (Figure 6C) but had no effect on bacterial adherence (data not shown).

Inhibition of c-Cbl expression could potentially affect signaling pathways involved in the entry process that are independent of Ku70. To address whether Ku70 ubiquitination is dependent on c-Cbl, we infected control and c-Cbl siRNA-treated HeLa cells and then determined the effects on Ku70 ubiquitination after *R. conorii* infection. As shown in Figure 6D, suppression of endogenous c-Cbl protein expression reduces the *R. conorii*-induced ubiquitination of Ku70. Together, these results demonstrate that the ubiquitin ligase Cbl plays an important role in the entry of *R. conorii* into nonphagocytic mammalian cells and, more importantly, that c-Cbl-mediated ubiquitination of Ku70 may contribute to *R. conorii* entry.

rOmpB Is the *R. conorii* Ligand that Interacts with Ku70

We were interested in determining the *R. conorii* ligand (or ligands) that could interact with Ku70 and stimulate bacterial entry. HEK293 cells were transfected with a plasmid encoding human FLAG-tagged Ku70 (pCMV2b-FLAG-Ku70FL), and FLAG-tagged Ku70 was purified by incubation with a FLAG-tag-specific affinity gel. As shown in Figure 7A, FLAG-Ku70FL copurifies with endogenous Ku70 and Ku80 when expressed in these cells. *R. conorii* protein extracts were

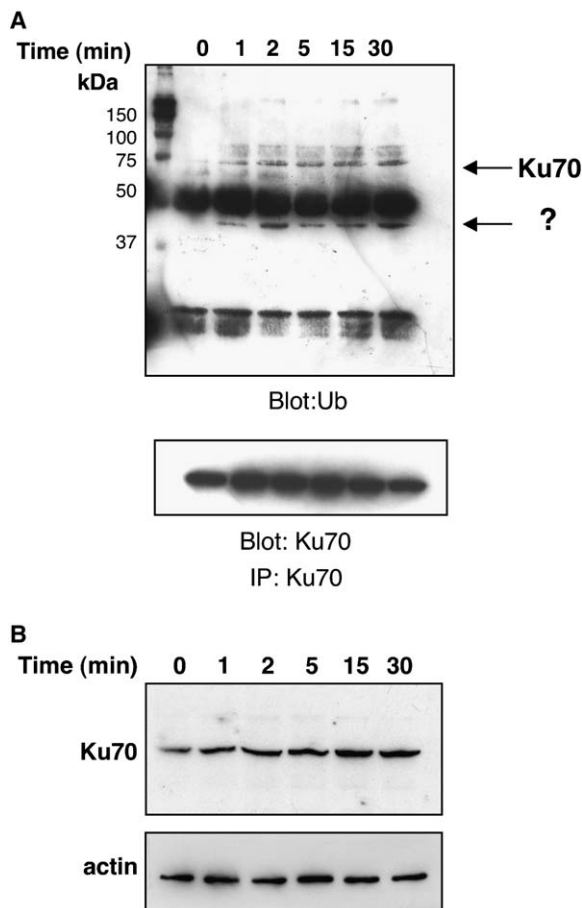


Figure 5. Ku70 Is Ubiquitinated in Response to *R. conorii* Infection

(A) HeLa cells were infected with *R. conorii* for the indicated times, and then Ku70 was immunoprecipitated from lysates from uninfected (time 0) and infected cells. Immunoblot analysis with anti-ubiquitin antiserum (Ub) reveals that Ku70 and at least one other unidentified protein (arrows) are ubiquitinated within minutes of bacterial infection of HeLa cells. The blot was stripped and reprobed with anti-Ku70 antisera to demonstrate equal Ku70 protein loading in each lane.

(B) Immunoblot analysis of total cell lysates from uninfected and *R. conorii*-infected HeLa cells demonstrates that Ku70 is not degraded in response to ubiquitination during *R. conorii* infection. Actin serves as a protein loading control.

incubated with FLAGM2 affinity gel coupled with FLAG-Ku70FL. As shown in Figure 7B, FLAG-Ku70FL specifically coeluted with endogenous Ku70 and Ku80 and with an approximately 150 kDa protein. Mass-spectrometry analysis of trypsin-digested peptides identified this protein as rOmpB, a SFG rickettsia protein belonging to a family of autotransporters in Gram-negative bacteria (reviewed in Henderson et al., 2004). rOmpB from a closely related SFG rickettsia, *R. japonica*, has been shown to mediate internalization in nonphagocytic Vero cells when expressed by noninvasive strains of *E. coli* (Uchiyama, 2003). Therefore, it is possible that the interaction between rOmpB expressed by *R. conorii* and Ku70 could mediate bacterial entry into nonphagocytic cells.

DISCUSSION

The invasion of target cells is critical for the subsequent growth and intracellular survival of *R. conorii*. We previously demonstrated that *R. conorii* induces signal transduction pathways within the infected cell to ultimately activate the actin-nucleating complex Arp2/3 (Martinez and Cossart, 2004). We demonstrate here that *R. conorii* specifically binds to Ku70, a component of the DNA-PK, and that the binding and recruitment of Ku70 at the plasma membrane are important events in the invasion of *R. conorii* into nonphagocytic mammalian cells.

Ku70 and Ku80/86 are the DNA binding components of the DNA-PK and have been shown to be involved in a variety of nuclear functions, including transcription regulation, chromosome maintenance, V(D)J recombination, and a specific DNA-repair mechanism called nonhomologous end-joining (NHEJ) (reviewed in Koike, 2002). Although primarily nuclear, Ku70 has been localized in the cytoplasm, where it can inhibit Bax-mediated apoptosis (Sawada et al., 2003), and also to the plasma membrane, where it can participate in heterologous and homologous cell adhesion (Koike, 2002) and fibronectin binding (Monferran et al., 2004a) and can interact with metalloprotease 9 (MMP-9) (Monferran et al., 2004b). Ku70 has also been found associated with lipid rafts, and it has been speculated that the presence of Ku70 within these domains may play an important role in signal transduction (Lucero et al., 2003). As for other invasive pathogens, including *L. monocytogenes* (Seveau et al., 2004), we demonstrated that entry of *R. conorii* into nonphagocytic cells was dependent on membrane cholesterol. We have confirmed the presence of Ku70 within DRMs, suggesting that Ku70 within lipid-raft domains may play an important role in initiating signals ultimately leading to bacterial entry.

How exactly Ku70 can be utilized by *R. conorii* at the plasma membrane to gain entry into cells is unclear. To initiate signaling events at the plasma membrane, Ku70 can either possibly associate with other proteins such as receptor tyrosine kinases (RTKs) or somehow signal through itself. Under certain conditions, the epidermal growth factor receptor, EGFR, can interact with DNA-PK and Ku70, which may be important for signaling events (Bandyopadhyay et al., 1998). Therefore, it is possible that Ku70-EGFR interactions may be important during the *R. conorii* entry process. However, we do not favor this model, as we could not detect an association between Ku70 and EGFR during the course of *R. conorii* infection. In addition, pharmacological inhibition of EGFR activation has no effect on *R. conorii* entry into nonphagocytic HeLa cells (J.J.M. and P.C., unpublished data).

Examination of structural motifs in the N termini of Ku70 and Ku80 revealed that these proteins share homology with integrin A domains and von Willebrand factor A1 and A3 domains that are involved in fibronectin binding (Monferran et al., 2004a). Ku70 and Ku80 contain residues that can form a metal-ion-dependent adhesion site (MIDAS), which is involved in ligand binding and activation of heterodimeric integrins, such as $\alpha 2 \beta 1$. We have previously demonstrated that *R. conorii* invasion into nonphagocytic mammalian cells involves the activation of src-family tyrosine kinases and the

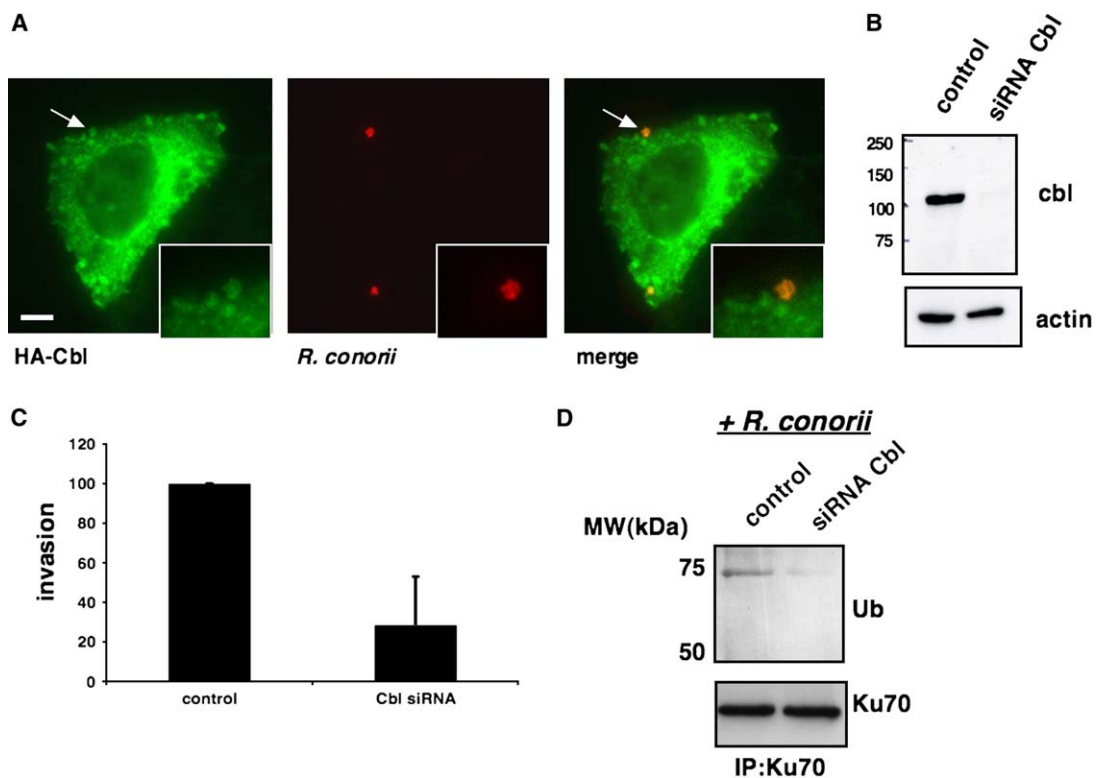


Figure 6. The Ubiquitin Ligase c-Cbl Is Involved in *R. conorii* Entry of Mammalian Cells and in the Ubiquitination of Ku70

(A) HeLa cells transfected with HA-c-Cbl were infected for 5 minutes with *R. conorii* and then processed for immunofluorescence. Extracellular *R. conorii* colocalizes with c-Cbl at sites of *R. conorii* attachment (arrow). The area indicated by the arrow is enlarged to show detail (boxed area). Bar represents 5 μ m. (B) siRNA against Cbl is able to efficiently block endogenous protein expression.

(C) Inhibition of Cbl expression suppresses *R. conorii* entry into HeLa cells.

(D) Control and siRNA-Cbl treated HeLa cells were infected with *R. conorii* for 10 minutes, cellular lysates were immunoprecipitated with anti-Ku70 antiserum, and immunoblots using anti-ubiquitin antiserum (Ub) were performed. Inhibition of endogenous Cbl expression inhibits Ku70 ubiquitination (Ub).

tyrosine phosphorylation of focal adhesion kinase (FAK) (Martinez and Cossart, 2004), two signaling events that are strongly associated with β 1-integrin activation (Parsons, 2003). In addition, the invasive pathogen *Yersinia enterocolitica* utilizes β 1-integrins to trigger its own uptake into nonphagocytic mammalian cells (Cossart and Sansonetti, 2004) in a process that shares some similarities with that of *R. conorii* (Martinez and Cossart, 2004). We thus favor a model in which binding of a specific *R. conorii* ligand (e.g., rOmpB; see below) to Ku70 would directly stimulate Ku70 at the membrane and lead to the induction of observed signaling cascades associated with bacterial entry (Martinez and Cossart, 2004). Alternatively, *R. conorii* could initially bind to fibronectin and utilize this interaction as a bridge to bind Ku70. Whether or not Ku70 can be utilized by *R. conorii* to mimic integrin signaling and ultimately lead to bacterial entry is currently being investigated.

Endocytosis is a cellular process by which proteins and macromolecules are internalized via the invagination and subsequent pinching off of membrane bound vesicles at the plasma membrane of mammalian cells (Conner and Schmid, 2003). The ligand-dependent endocytosis of several host-cell membrane proteins, including RTKs, is in some cases driven by the Cbl-dependent monoubiquitination of the re-

ceptor (Haglund et al., 2003; Marmor and Yarden, 2004). It is well established that the endocytic process is exploited by viral pathogens to enter nonphagocytic target cells (Sieczkarski and Whittaker, 2005), and recent data from our laboratory revealed that the clathrin-dependent endocytic machinery plays a crucial role in the entry of *L. monocytogenes* into nonphagocytic cells (Veiga and Cossart, 2005). We have demonstrated here that internalization of *R. conorii* into mammalian cells correlates with the rapid and sustained ubiquitination of Ku70 and that c-Cbl plays a critical role in the bacterial entry process. In addition, we have shown that the *R. conorii*-mediated ubiquitination of Ku70 was inhibited in cells with reduced levels of endogenous c-Cbl, suggesting that c-Cbl is the ubiquitin ligase that modifies Ku70 during bacterial entry. Whether other components of the endocytic machinery are involved is under investigation.

We have previously demonstrated the host actin polymerization and the activity of the Arp2/3 complex is crucial in the *R. conorii* entry pathway. Interestingly, recent evidence has shown that the actin cytoskeleton of mammalian cells plays a crucial role in the endocytic process (Engqvist-Goldstein and Drubin, 2003; Yarar et al., 2005). *R. conorii* interactions with Ku70 and possibly other proteins could generate a signaling cascade ultimately leading to the activation of the

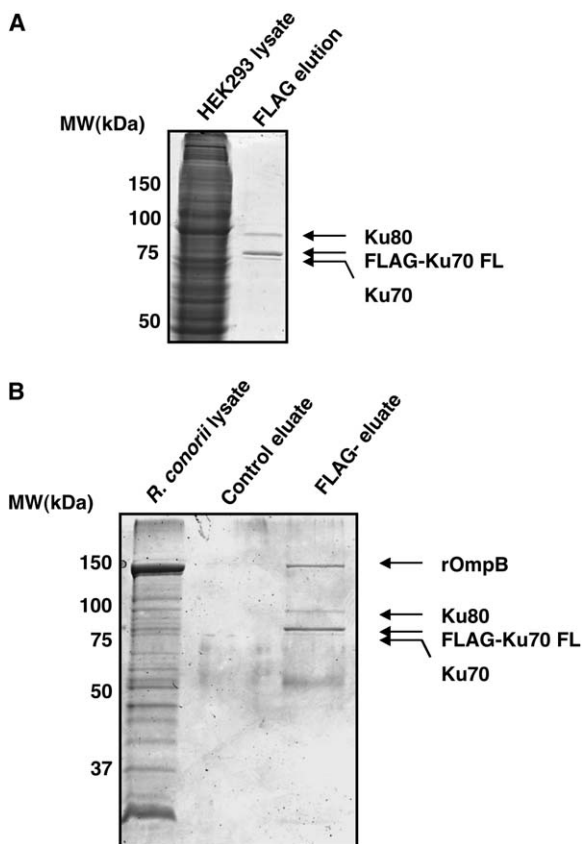


Figure 7. The rOmpB Protein from *R. conorii* Interacts with Ku70

(A) In HEK293 cells, transfected FLAG-Ku70FL copurifies with endogenous Ku70 and Ku80 when purified by FLAG affinity chromatography (arrows). HEK293 lysate represents the cell lysate prior to purification, while FLAG elution refers to the 3 \times FLAG peptide elution of purified proteins. (B) Proteins isolated by detergent extraction (*R. conorii* lysate) were incubated with the FLAGM2 affinity gel or FLAGM2 affinity gel coupled with FLAG-Ku70FL (and copurified endogenous Ku70 and Ku80). As identified by mass spectrometry, rOmpB copurifies with FLAG-Ku70FL (FLAG eluate, arrows). The interaction of rOmpB with Ku70 is specific and is not due to interactions with the FLAGM2 affinity gel (control eluate).

Arp2/3 complex and resulting in localized actin polymerization observed during bacterial entry (Martinez and Cossart, 2004). *R. conorii* could stimulate Ku70 ubiquitination and potentially “hijack” the endocytic machinery, which, coupled with Arp2/3-dependent actin polymerization, would contribute to the internalization of *R. conorii* into nonphagocytic cells.

Ku70 is involved in a variety of cellular functions that could potentially be utilized by *R. conorii* during the infection process. Interestingly, Ku70 in the cytoplasm functions as a potent inhibitor of Bax-mediated apoptosis, resulting in the maintenance of mitochondrial integrity and the inhibition of downstream effector caspases (Sawada et al., 2003). Apoptosis of infected cells could be a mechanism utilized by the host to eliminate the intracellular infection (Cossart and Sansonetti, 2004). However, obligate intracellular pathogens, such as SFG rickettsiae, depend on host-cell viability

to ensure survival and growth and have evolved mechanisms to subvert apoptotic mechanisms within the infected cell (Clifton et al., 1998). As shown recently, infection of endothelial cells with *R. rickettsii*, an SFG member closely related to *R. conorii*, results in the inhibition of caspase activation and the maintenance of mitochondrial integrity through pathways involving NF- κ B (Joshi et al., 2003). Preliminary results suggest that pathways involving Ku70 and Bax may also contribute to the maintenance of mitochondrial integrity during prolonged *R. conorii* infections of cultured cells (J.J.M. and P.C., unpublished data). The putative role of Ku70 in the inhibition of apoptosis during *R. conorii* infection of host cells warrants further investigation.

We show here that Ku70 interacts with rOmpB, which is the most abundant surface protein expressed by rickettsiae and is responsible for an arrayed surface structure called the S layer (Carl et al., 1990). Interestingly, rOmpB exhibits homology to a family of modular proteins in Gram-negative bacteria called autotransporters, many of which have been shown to be important for virulence (reviewed in Henderson et al., 2004). rOmpB is expressed as a 168 kDa protein and, as with other autotransporters, is cleaved to release a 135 kDa domain from a translocation pore (32 kDa), leaving the mature 135 kDa domain associated with the outer leaflet of the outer membrane (Hackstadt et al., 1992). Defects in the ability to process rOmpB correlated with a decrease in virulence in certain SFG rickettsiae (Hackstadt et al., 1992). rOmpB expressed by *R. japonica*, a SFG rickettsiae closely related to *R. conorii*, is sufficient to mediate binding and entry of nonphagocytic Vero cells when expressed in noninvasive *E. coli* (Uchiyama, 2003). Furthermore, anti-rOmpB antibodies protect mice from an otherwise lethal challenge of *R. conorii* (Feng et al., 2004a, 2004b) in a model of Mediterranean spotted fever, suggesting that rOmpB may play a crucial role in the pathogenesis of SFG rickettsial infections. Whether the interaction between rOmpB expressed by *R. conorii* and Ku70 is necessary and sufficient to mediate bacterial entry is currently being investigated.

Our results do not exclude the possibility that other unidentified host-cell proteins and cognate bacterial ligands could also be involved in the entry process. For instance, another outer-membrane protein, rOmpA (Li and Walker, 1998), expressed by SFG rickettsiae has been proposed to mediate adherence to and putative invasion of host cells. However, the host-cell receptor (or receptors) interacting with rOmpA is currently not known. In the light of the potential use of rickettsial species as agents of biological terrorism, a further understanding of the complex interplay between the host and pathogen is of great importance and could lead to the development of novel, efficacious therapies in the treatment and prevention of rickettsial diseases.

EXPERIMENTAL PROCEDURES

Cell Lines and Bacterial Strains

The African green monkey kidney epithelial cell line, Vero (ATCC CRL 1587), HEK293 cells, and HeLa cells were cultured as described (Martinez and Cossart, 2004). Mouse embryonic fibroblasts (MEFs) derived from *Ku70*^{+/+} and *Ku70*^{-/-} mouse strains were cultured as described (Sawada et al., 2003). *R. conorii* was grown in Vero cells as previously

described (Martinez and Cossart, 2004). *L. monocytogenes* strain EGD (BUG600) was grown in brain-heart infusion (BHI) broth at 37°C, while *E. coli* DH5 α and *S. flexneri* (strain M90T, Institut Pasteur) were grown in LB broth at 37°C. Bacteria were diluted 1:10 from overnight cultures, grown to an OD₆₀₀ of 0.8, and washed in sterile 1 \times PBS before use.

Antibodies and Other Reagents

Mouse monoclonal antibody (mAb) against actin (clone AC-40), polyclonal horseradish peroxidase (HRP) conjugated anti-mouse IgG, and anti-rabbit IgG antisera used for immunoblotting; HRP-conjugated streptavidin; FLAG-tagged Protein Immunoprecipitation kit; methyl- β -cyclodextrin (M β CD); and water-soluble cholesterol were obtained from Sigma. For immunofluorescence studies, AlexaFluor 488- and AlexaFluor 546-conjugated goat anti-rabbit IgG and goat anti-mouse IgG antisera and Cy3-conjugated anti-goat IgG antiserum were purchased from Molecular Probes. Rabbit polyclonal anti-*R. conorii* antiserum (R47) has been described (Gouin et al., 1999). Goat polyclonal anti-Ku70 (C-19), anti-HA epitope tag mAb (F-7), and isotype-matched IgG_{2b} antisera (PY20) were purchased from Santa Cruz Biotechnology. Anti-Ku70 mAb (N3H10) was purchased from NeoMarkers (Fremont, CA). Rabbit polyclonal anti-caveolin-2 and anti-c-Cbl mAb antisera were obtained from BD Transduction Laboratories. Anti-ubiquitin mAb (P4D1) was purchased from Cell Signaling Technologies. Brij-58, Triton X-100 (10% stock solutions), non-membrane-permeable sulfo-NHS-biotin reagent, and SuperSignal WestPico enhanced chemiluminescence system were purchased from Pierce. Protein A and Protein G Sepharose were purchased from Pharmacia. Membrane blocking solution for Western immunoblots was purchased from Zymed. Complete protease inhibitor cocktail was purchased from Boehringer Mannheim. Annealed small interfering RNAs (siRNAs) against Ku70 sense strand 5'-GGAAGAGAUAGUUGAUUUTT-3', anti-sense strand 5'-AAAUCAAACUUAUCUCUUCCTG-3', and Silencer negative control #1 siRNA were purchased from Ambion. siRNA against c-Cbl has been described (Veiga and Cossart, 2005). A plasmid coding for HA-tagged c-Cbl (pMT2SM-HA-c-Cbl) was kindly provided by Dr. Jannie Borst (Taher et al., 2002). A plasmid coding for FLAG-epitope-tagged human Ku70 in pCMV-2B (pCMV-2B FLAG Ku70FL) has been described (Sawada et al., 2003).

Identification of *R. conorii* Host-Cell Receptors (Bacterial Affinity Pull-down Assays)

1.5 \times 10⁸ plaque-forming units (pfu) of *R. conorii* and 1.0 \times 10⁹ colony-forming units (cfu) each of *E. coli*, *S. flexneri*, and *L. monocytogenes* were pelleted and washed in 1 \times PBS. HeLa and Vero whole-cell lysates were prepared from approximately 1.0 \times 10⁷ cells (100 mm dish of confluent cells) by lysing cells in 500 μ l of 1% NP-40 lysis buffer as described (Martinez and Cossart, 2004). In some experiments, host cells were incubated with 0.5 mM sulfo-NHS-biotin to label surface proteins and unreacted biotin was quenched with excess glycine (1 M) according to manufacturer's directions (Pierce). Cellular extracts were incubated with washed bacterial pellets at 4°C for 2 hr, centrifuged at 8000 \times g for 5 min at 4°C, and washed in 1% NP-40 lysis buffer. Putative receptors were eluted by incubation at 4°C for 1 hr in 1% NP-40 lysis buffer containing an additional 1.0 M NaCl. Eluted proteins were recovered via centrifugation, separated by 10% SDS-PAGE, and visualized by silver staining. Protein identification by mass spectrometry was performed (Proteomic Platform, Genopole, Institut Pasteur, Paris) as described (Saveanu et al., 2003).

To visualize biotin-labeled surface proteins that interacted with bacteria, eluted proteins were separated on 10% SDS-PAGE, transferred to nitrocellulose, and incubated with HRP-labeled streptavidin (1:5000) in membrane blocking buffer (Zymed). In some experiments, washed *R. conorii* bacterial pellets (1.5 \times 10⁸ pfu) were boiled in 2 \times SDS-PAGE sample buffer and used as control to verify purity of bacterial preparation (*R. conorii* lysate). Results shown are representative of two independent assays.

RNA Interference (RNAi)

HeLa cells were plated onto six-well plates at 1.2 \times 10⁵ cells per well and transfected with the indicated siRNAs as described (Veiga and Cossart,

2005). Forty-eight hours posttransfection, cells were harvested by trypsinization and replated for an additional 24 hr onto sterile glass coverslips in 24-well plates (1.2 \times 10⁵ cells per coverslip) for bacterial internalization assays as described below or six-well tissue culture plates (3.5 \times 10⁵ cells per well) for subsequent Western immunoblot analysis of protein expression as described below.

Bacterial Internalization Assay

R. conorii internalization assays were performed as described (Martinez and Cossart, 2004). For antibody inhibition experiments, mammalian cells were preincubated with 15 μ g/ml of mouse monoclonal anti-Ku70 (N3H10) antiserum, mouse monoclonal isotype-matched control antiserum (PY20), and goat polyclonal anti-Ku70 (C-19) antiserum for 30 min in serum-free DMEM prior to the addition of bacteria. *L. monocytogenes* gentamicin survival assays were performed as described (Mengaud et al., 1996). Results are presented as invasion and adherence percentages relative to untransfected or untreated control cells (set to 100%) and were calculated as described (Martinez and Cossart, 2004). Data shown are representative of at least two independent experiments.

Cholesterol Depletion and Repletion of Cells

Cholesterol depletion and repletion of mammalian cells were performed essentially as described (Seveau et al., 2004), and assays were performed as indicated. Isolation of DRMs via sucrose-density-gradient centrifugation was performed as described (Seveau et al., 2004). Proteins in each fraction were separated by 10% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with the indicated antisera.

Immunofluorescence Studies and Transient Cell Transfection

Vero and HeLa cells were seeded onto sterile 12 mm glass coverslips in 24-well plates, infected for 15 min with *R. conorii*, and then processed for immunofluorescence as described (Martinez and Cossart, 2004) using rabbit anti-*R. conorii* (R47) and AlexaFluor 488-conjugated goat anti-rabbit IgG antibodies. In some experiments, endogenous Ku70 was visualized in permeabilized and nonpermeabilized cells with 1 μ g/ml anti-Ku70 mAb (clone N3H10) and AlexaFluor 546-conjugated anti-mouse IgG or 0.5 μ g/ml anti-Ku70 (C-19) and Cy3-conjugated anti-goat IgG.

HeLa cells were transfected with 1.0 μ g of the indicated DNA, infected with *R. conorii* (multiplicity of infection \sim 20) for 1–5 min, and fixed in paraformaldehyde (PFA) as described (Martinez and Cossart, 2004). Fixed cells were processed for immunofluorescence using rabbit anti-*R. conorii* antisera (R47, 1:500) and AlexaFluor 546 goat-anti-rabbit IgG to label bacteria. Transfected cells were identified using anti-HA epitope antiserum (F-7, 1:500) and AlexaFluor 488 anti-mouse IgG (1:500) antiserum.

Immunoprecipitations and Western Immunoblotting

Infections of control and siRNA-treated cells and subsequent immunoprecipitations were performed as described (Martinez and Cossart, 2004) using anti-Ku70 antisera (1 μ g C-19/sample). Western immunoblotting using anti-ubiquitin (P4D1, 1 μ g/ml) has been described (Veiga and Cossart, 2005). In some experiments, equal amounts of protein from control and siRNA-treated cells were separated on 10% SDS-PAGE, transferred to nitrocellulose, and blotted with the indicated antisera. Blots were stripped with Restore membrane stripping solution (Pierce) and re-blotted with the indicated antisera to demonstrate that equal amounts of protein were loaded in each lane. Blots shown are representative of at least two independent experiments.

Identification of *R. conorii* Proteins Interacting with Ku70

1.5 \times 10⁸ plaque-forming units (pfu) of *R. conorii* were washed in 1 \times PBS, and then bacterial extracts were made using B-PERII Bacterial Protein Extraction Reagent according to manufacturer's instructions (Pierce). HEK293 cells were left untransfected (control) or were transfected with 20 μ g of pCMV-2B FLAG Ku70FL using the Lipofectamine 2000 reagent according to manufacturer's instructions (Invitrogen). Cellular lysates were prepared 48 hr posttransfection from transfected and nontransfected cells using the FLAG-tagged Protein Immunoprecipitation kit (Sigma) according to manufacturer's instructions with certain modifications. Briefly,

lysates from untransfected and transfected cells were incubated with 100 μ l of the anti-FLAGM2-agarose affinity gel overnight at 4°C. Gels were washed extensively in 1 \times wash buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl), and then the affinity gel incubated with FLAG-tagged Ku70 was divided into two 50 μ l aliquots. One aliquot was saved to determine the purity of the FLAG-Ku70 purification, while the other aliquot was incubated with approximately 750 μ g of *R. conorii* extracts for 2 hr at 4°C. The affinity gels were then washed extensively in 1 \times wash buffer, and FLAG-tagged Ku70 as well as proteins specifically interacting with Ku70 were eluted by incubating the affinity gels in 1 \times wash buffer containing 150 ng/ μ l of 3 \times FLAG peptide (Sigma). Eluted proteins were separated by 10% SDS-PAGE and stained with Coomassie blue, and then proteins of interest were identified by mass spectrometry (Proteomic Platform, Genopole, Institut Pasteur) as described (Saveanu et al., 2003).

Supplemental Data

Supplemental Data include two figures and can be found with this article online at <http://www.cell.com/cgi/content/full/123/6/1013/DC1/>.

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